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(57) Abstract

The present invention relates generally to methods for treating sensory neuropathy by administering glial cell line-derived neurotrophic factor (GDNF).

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METHOD FOR TREATING SENSORY NEUROPATHY USING GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) PROTEIN PRODUCT

BACKGROUND OF THE INVENTION

The present invention relates generally to methods for treating sensory neuropathy, by administering glial cell line-derived neurotrophic factor (GDNF) protein product.

Neurotrophic factors are natural proteins, found in the nervous system or in non-nerve tissues innervated by the nervous system, that function to promote the survival and maintain the phenotypic differentiation of certain nerve and/or glial cell populations (Varon et al., *Ann. Rev. Neuroscience*, 1:327, 1979; Thoenen et al., *Science*, 229:238, 1985). Because of this physiological role, neurotrophic factors are useful in treating the degeneration of such nerve cells and the loss of differentiated function that results from nerve damage. Nerve damage is caused by conditions that compromise the survival and/or proper function of one or more types of nerve cells, including: (1) physical injury, which causes the degeneration of the axonal processes (which in turn causes nerve cell death) and/or nerve cell bodies near the site of injury, (2) temporary or permanent cessation of blood flow (ischemia) to parts of the nervous system, as in stroke, (3) intentional or accidental exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatinum and dideoxycytidine , respectively, (4) chronic metabolic diseases, such as diabetes or renal dysfunction, or (5) neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis, which result from the degeneration of specific neuronal populations. In order for a particular neurotrophic factor to be potentially useful in treating nerve damage, the class or classes of damaged nerve cells must be responsive to the factor. It has been established that all neuron populations are not responsive to or equally affected by all neurotrophic factors.

The first neurotrophic factor to be identified was nerve growth factor (NGF). NGF is the first member of a defined family of trophic factors, called the neurotrophins, that currently includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (Thoenen, *Trends. Neurosci.*, 14:165-170, 1991; Snider, *Cell*, 77:627-638, 1994; Bothwell, *Ann.*

Rev. Neurosci., 18:223-253, 1995). These neurotrophins are known to act via the family of *trk* tyrosine kinase receptors, i.e., *trk*A, *trk*B, *trk*C, and the low affinity p75 receptor (Snider, *Cell*, 77:627-638, 1994; Bothwell, *Ann. Rev. Neurosci.*, 18:223-253, 1995; Chao et al., *TINS* 18:321-326, 1995). NGF is the classic molecule for sensory neurons (Theonen and Barde, *Physiol. Rev.*, 60:1284-335, 1980) and has been demonstrated recently to be effective in the prevention of taxol-induced small fiber neuropathy (Apfel et al., *Ann. Neurol.*, 29:87-90, 1991), cisplatin-induced large fiber neuropathy (Apfel et al., *Ann. Neurol.*, 31:76-80, 1992), and experimental diabetic neuropathy (Apfel et al., *Brain Res.*, 634:7-12, 1994) in animal models.

Glial cell line-derived neurotrophic factor (GDNF) is a recently discovered protein identified and purified using assays based upon its efficacy in promoting the survival and stimulating the transmitter phenotype of mesencephalic dopaminergic neurons in vitro (Lin et al., *Science*, 260:1130-1132, 1993). GDNF is a glycosylated disulfide-bonded homodimer that has some structural homology to the transforming growth factor-beta (TGF-ß) super family of proteins (Lin et al., *Science*, 260:1130-1132, 1993; Krieglstein et al., *EMBO J.*, 14:736-742, 1995; Poulsen et al., *Neuron*, 13:1245-1252, 1994). GDNF mRNA has been detected in muscle and Schwann cells in the peripheral nervous system (Henderson et al., *Science*, 266:1062-1064, 1994; Trupp et al., *J. Cell Biol.*, 130:137-148, 1995) and in type I astrocytes in the central nervous system (Schaar et al., *Exp. Neurol.*, 124:368-371, 1993). *In vivo*, treatment with exogenous GDNF stimulates the dopaminergic phenotype of substantia nigra neurons and restores functional deficits induced by axotomy or dopaminergic neurotoxins in animal models of Parkinson's disease (Hudson et al., *Brain Res. Bull.*, 36:425-432, 1995; Beck et al., *Nature*, 373:339-341, 1995; Tomac et al., *Nature*, 373:335-339, 1995; Hoffer et al., *Neurosci. Lett.*, 182:107-111, 1994). Although originally thought to be relatively specific for dopaminergic neurons, at least *in vitro*, evidence is beginning to emerge indicating that GDNF may have a larger spectrum of neurotrophic targets besides mesencephalic dopaminergic and somatic motor neurons (Yan and Matheson, *Nature*, 373:341-344, 1995; Oppenheim et al., *Nature*, 373:344-346, 1995; Matheson et al., *Soc. Neurosci. Abstr.*, 21, 544, 1995; Trupp et al., *J. Cell Biol.*, 130:137-148, 1995). In particular, GDNF was found to have neurotrophic efficacy on brainstem and spinal cord cholinergic motor neurons, both *in vivo* and *in vitro* (Oppenheim et al., *Nature*, 373:344-346, 1995; Zurn et

al., *Neuroreport*, 6:113-118, 1994; Yan et al., *Nature*, 373: 341-344, 1995; Henderson et al., *Science*, 266:1062-1064, 1994).

Sensory neuropathy is a relatively common neurological condition which can occur spontaneously or as a secondary complication of trauma or other diseases such as diabetes mellitus, HIV infection, and cancer chemotherapy. About 66% of diabetes mellitus patients develop sensory neuropathy, and about 7% of HIV-infected patients develop sensory neuropathy every year. For cancer patients, the dose of chemotherapeutic agent used is often limited by the development of toxic neuropathies. Sensory neuropathy can result in neuropathic pain, loss of external sensation, or the loss of protective sensation such as nociceptive, vibratory and proprioceptive perception.

Two major somatic sensory pathways serve both exteroception (perception of environmental influences, including pressure, touch, temperature and pain) and conscious proprioception (including vestibular system perception, joint position sense and muscle sensation). Sensory neurons innervating the head reside in cranial ganglia, and they directly project to the sensory nuclei in the brain stem. All primary sensory afferents of the rest of the body have their cell bodies in the dorsal root ganglia. The two different sensory pathways are anatomically separated and have different synapse patterns. The first pathway relays sensations of pain, temperature and crude touch and begins as free nerve endings of small myelinated and unmyelinated fibers. These small fibers of the first pathway enter the spinal cord via the dorsal root ganglia, lateral to the large myelinated fibers of the second pathway, and then ascend or descend for one or two segments before synapsing in the dorsal horn and thereafter ascending to the brain. The second pathway consists predominantly of larger myelinated fibers and relays sensations of light touch, position sense and tactile localization. These large fibers also enter the spinal cord via the dorsal root ganglia, and lie in a position medial to the smaller fibers of the first pathway.

Most disorders of the peripheral somatosensory system occur in the axon itself or in the myelin sheath encasing the axon. Selective small fiber damage is sometimes encountered in diabetes and is common in some of the hereditary neuropathies as well as in toxic-nutritional neuropathies. Large fiber loss is more common in demyelinating neuropathies and may lead to profound loss of touch and proprioception. Such disorders are commonly accompanied by a loss of sensation and sometimes pain. Mononeuropathies are generally caused by local disease (such as compression entrapment or other trauma,

ischemia, tumor or granulomatous infiltration, amyloid deposition, leprosy, etc.). Vascular mononeuritis result from vascular disorders, such as polyarteritis. Symmetric generalized polyneuropathies result from immunologic or metabolic disorders (for example, demyelination or inflammatory neuropathies, diabetes, toxin or drug-induced injury, and metabolic diseases such as uremia or nutritional deficiency syndromes).

Acute inflammatory sensory polyneuropathy involves an inflammatory response that is usually associated with a preceding infection, typically one occurring several weeks earlier. The diabetic neuropathies include isolated mononeuropathies and symmetrical polyneuropathies, which generally manifest with distal sensory loss. The cause of diabetic neuropathies is unknown but a metabolic basis has been suggested. Uremic polyneuropathy can be associated with chronic renal insufficiency of any type; the cause is unknown but is presumed to be related to toxins or other metabolites normally excreted by the kidneys. Hypothyroidism is associated with mononeuropathy and symmetrical polyneuropathy. Serum monoclonal gammopathies are also associated with polyneuropathy. Acromegaly produces entrapment neuropathies in the limbs and a distal symmetrical polyneuropathy. Cancer-associated mononeuropathies can be caused by direct compression or infiltration of nerves by tumors; polyneuropathy can be caused by multiple myeloma. Other vascular-related neuropathies (caused by vasculitis or ischemia) are associated with various conditions such as polyarteritis nodosa, rheumatoid arthritis, systemic lupus erythematosus, hypersensitivity angiitis, allergic granulomatosis, Sjogren's syndrome, Wegener's granulomatosis and cranial arteritis. Infectious and granulomatous neuropathies include those associated with Lyme disease, HIV infection, herpes zoster infection and leprosy. The hereditary sensory neuropathies, types I, II and III, are a group of slowly progressive disorders probably caused by inborn errors of metabolism. Toxic neuropathies are caused by pharmaceutical or chemical agents, such as chloramphenicol, dapsone, disulfiram, dichloracetate, ethionamide, gold, glutethimide, hydralazine, isoniazid, lithium, metronidazole-misonidazole, nitrofurantoin, nitrous oxide, platinum, cis-platinum, pyridoxine, sodium cyanate, thalidomide, vincristine, acrylamide, arsenic, buckthorn toxin, carbon disulfide, cyanide, dimethylaminoproprionitrile, dichlorophenoxyacetic acid, diptheria toxin, ethylene oxide, hexacarbons (n-hexane, methyl n-butyl ketone), lead, Lucel-7, methyl bromide, organophosphates, thallium, and trichlorethylene. Alcoholism is also associated with neuropathy.

Many neuropathies are treated with corticosteroids, but there are currently no specific treatments for sensory neuropathies. Other growth factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3), and insulin growth factor-I (IGF-I) are reportedly in clinical trials for treatment of peripheral neuropathies. These agents, however, may cause undesirable side effects and toxicity.

Of general interest to the present invention is WO93/06116 (Lin et al., Syntex-Synergen Neuroscience Joint Venture), published April 1, 1993, which reports that GDNF is useful for the treatment of nerve injury, including injury associated with Parkinson's disease. Also of interest are a report in Schmidt-Kastner et al., *Mol. Brain Res.*, 26:325-330, 1994 that GDNF mRNA became detectable and was upregulated after pilocarpine-induced seizures; Schaar et al., *Exp. Neurol.*, 124:368-371, 1993 and Schaar et al., *Exp. Neurol.*, 130:387-393, 1994 report that basal forebrain astrocytes expressed moderate levels of GDNF mRNA under culture conditions, but that GDNF did not alter basal forebrain ChAT activity; and a report in currently pending U.S. Application Serial No. 08/535,682 filed September 28, 1995 that GDNF is useful for treating injury or degeneration of basal forebrain cholinergic neurons.

Of particular interest to the present invention is the disclosure in Henderson et al., *Science*, 266:1062-1064, 1994 that GDNF had no effect on the survival of embryonic peripheral sensory neurons in culture. There continues to exist a need for methods and therapeutic compositions useful for the treatment of sensory neuropathy. Such methods and therapeutic compositions would ideally protect sensory neurons from progressive injury and promote the survival or regeneration of damaged sensory neurons, without severe adverse side effects.

SUMMARY OF THE INVENTION

The present invention provides methods for treating sensory neuropathy caused by injury to, insults to, or degeneration of, sensory neurons, by administering a therapeutically effective amount of glial cell line-derived neurotrophic factor (GDNF) protein product. The sensory neuropathy may be a secondary complication of a non-neurological condition, such as trauma. It is contemplated that such GDNF protein products would include a GDNF protein such as that depicted by the amino acid sequence set forth in SEQ ID NO:1, as

well as variants and derivatives thereof. The invention is based on the finding that sensory neurons selectively take up and retrogradely transport GDNF protein product, and the novel discovery that GDNF enhances the survival of injured sensory neurons.

According to one aspect of the present invention, the GDNF protein product may be administered parenterally at a dose ranging from about 1 μg/kg/day to about 100 mg/kg/day, typically at a dose ranging from about 1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day to 20 mg/kg/day. GDNF protein product is preferably administered directly subcutaneously or intramuscularly close to or at the site of injury or degeneration; in such cases, a smaller dose of GDNF protein product, from about 1 μg/kg to 1 mg/kg, will be administered, for example, 1 μg/kg to 1 mg/kg administered at intervals ranging from weekly to daily . It is further contemplated that the GDNF protein product be administered in combination or conjunction with an effective amount of a second therapeutic agent for the treatment of sensory neuropathy.

The invention also provides for the use of GDNF protein product in the manufacture of a medicament or pharmaceutical composition for the treatment of sensory neuropathy. Such pharmaceutical composition include topical, oral or parenteral GDNF protein product formulations. It will also be appreciated by those skilled in the art that the administration process can be accomplished via cell therapy and gene therapy means, as further described below. Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently preferred embodiments thereof.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for treating sensory neuropathy caused by injury to, insults to, or degeneration of, sensory neurons, by administering a therapeutically effective amount of glial cell line-derived neurotrophic factor (GDNF) protein product by means of a pharmaceutical composition, the implantation of GDNF-expressing cells, or GDNF gene therapy. The invention may be practiced using any biologically active GDNF protein product, including a GDNF protein represented by the amino acid sequence set forth in SEQ ID NO:1, including variants and derivatives thereof.

In addition to oral, parenteral or topical delivery of the GDNF protein product, administration via cell therapy and gene therapy procedures is contemplated.

The present invention is based on the finding that GDNF protein product is selectively taken up and retrogradely transported to the cell body of sensory neurons through a receptor-mediated mechanism. It was then discovered that GDNF protein product serves as a survival factor for sensory neurons as demonstrated in the neonatal axotomy-induced cell death paradigm. After injection into the hindlimb of neonatal rats or the sciatic nerve of adult rats, GDNF protein product was retrogradely transported to sensory neurons in the lumbar dorsal root ganglia in a receptor-mediated fashion. In the neonatal sciatic nerve axotomy model, which usually results in about 50% neuronal cell death in the lumbar dorsal root ganglia without treatment, local application of GDNF protein product to the cut sciatic nerve rescued nearly 100% of the sensory neurons from axotomy-induced cell death. The survival-promoting effect of GDNF protein product on neonatal dorsal root ganglia neurons indicates that this factor has therapeutic value in the treatment of sensory neuropathy.

Due to the survival-promoting effects on sensory neurons which is demonstrated herein, it is contemplated that treatment with GDNF protein product will be advantageous in treating patients suffering from sensory neuropathy which can occur spontaneously or as a secondary complication of trauma, infection, inflammatory response, or other diseases or conditions, such as diabetes mellitus, immunologic or metabolic disorders and cancer, as described above. Similarly, GDNF protein product therapy may be advantageous in treating the hereditary sensory neuropathies, nutritional deficiency syndromes and toxic neuropathies, as described above. In addition, GDNF protein product therapy may be advantageous in treating vascular-related neuropathies (caused by vasculitis or ischemia) associated with various conditions such as polyarteritis nodosa, rheumatoid arthritis, systemic lupus erythematosus, hypersensitivity angiitis, allergic granulomatosis, Sjogren's syndrome, Wegener's granulomatosis and cranial arteritis.

According to the invention, the GDNF protein product may be administered parenterally at a dose ranging from about 1 μg/kg/day to about 100 mg/kg/day, typically at a dose of about 1 mg/kg/day to about 25 mg/kg/day, and usually at a dose of about 5 mg/kg/day and 20 mg/kg/day. GDNF protein product is preferably administered directly subcutaneously or intramuscularly close to the site of injury or degeneration. It is also contemplated that GDNF

protein product may be administered subcutaneously or intramuscularly at a dose of about 1 μg/kg/day to 1 mg/kg/day. It is further contemplated that GDNF protein product may be administered subcutaneously or intramuscularly at a dose of 1 μg/kg to 10 mg/kg once per week or several times per week. The GDNF protein product also may be administered in combination or conjunction with an effective amount of a second therapeutic agent for the treatment of sensory neuropathy, such as NGF, NT-3 and IGF-I. The invention also provides for the use of GDNF protein product in the preparation of a medicament for the treatment of sensory neuropathy. A variety of pharmaceutical formulations and different delivery techniques are described in further detail below.

As used herein, the term "GDNF protein product" includes purified natural, synthetic or recombinant glial cell line-derived neurotrophic factor, biologically active GDNF variants (including insertion, substitution and deletion variants), and chemically modified derivatives thereof. Also included are GDNFs that are substantially homologous to the human GDNF having the amino acid sequence set forth in SEQ ID NO:1. GDNF protein products may exist as homodimers or heterodimers in their biologically active form.

The term "biologically active" as used herein means that the GDNF protein product demonstrates similar neurotrophic properties, but not necessarily all of the same properties, and not necessarily to the same degree, as the GDNF having the amino acid sequence set forth in SEQ ID NO:1. The selection of the particular neurotrophic properties of interest depends upon the use for which the GDNF protein product is being administered.

The term "substantially homologous" as used herein means having a degree of homology to the GDNF having the amino acid sequence set forth in SEQ ID NO:1 that is preferably in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90% or 95%. For example, the degree of homology between the rat and human protein is about 93%, and it is contemplated that preferred mammalian GDNF will have a similarly high degree of homology. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to assist in that alignment (as set forth by Dayhoff, in *Atlas of Protein Sequence and Structure*, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), the disclosure of which is hereby incorporated by

reference). Also included as substantially homologous is any GDNF protein product which may be isolated by virtue of cross-reactivity with antibodies to the GDNF of SEQ ID NO:1 or whose genes may be isolated through hybridization with the gene or with segments of the gene encoding the GDNF of SEQ ID NO:1.

The GDNF protein products according to this invention may be isolated or generated by any means known to those skilled in the art. Exemplary methods for producing GDNF protein products useful in the present invention are described in U.S. Patent Application Serial No. 08/182,183 filed May 23, 1994 and its parent applications; PCT Application No. PCT/US92/07888 filed September 17, 1992, published as WO 93/06116 (Lin et al., Syntex-Synergen Neuroscience Joint Venture); European Patent Application No. 92921022.7, published as EP 610 254; and co-owned, co-pending U.S. Patent Application Serial No. 08/535,681 filed September 28, 1995 ("Truncated Glial Cell-Line Derived Neurotrophic Factor,"), the disclosures of which are hereby incorporated by reference.

Naturally-occurring GDNF protein products may be isolated from mammalian neuronal cell preparations, or from a mammalian cell line secreting or expressing GDNF. For example, WO93/06116 describes the isolation of GDNF from serum-free growth conditioned medium of B49 glioblastoma cells. GDNF protein products may also be chemically synthesized by any means known to those skilled in the art. GDNF protein products are preferably produced via recombinant techniques because they are capable of achieving comparatively higher amounts of protein at greater purity. Recombinant GDNF protein product forms include glycosylated and non-glycosylated forms of the protein, and protein expressed in bacterial, mammalian or insect cell systems.

In general, recombinant techniques involve isolating the genes responsible for coding GDNF, cloning the gene in suitable vectors and cell types, modifying the gene if necessary to encode a desired variant, and expressing the gene in order to produce the GDNF protein product. Alternatively, a nucleotide sequence encoding the desired GDNF protein product may be chemically synthesized. It is contemplated that GDNF protein product may be expressed using nucleotide sequences which differ in codon usage due to the degeneracies of the genetic code or allelic variations. WO93/06116 describes the isolation and sequencing of a cDNA clone of the rat GDNF gene, and the isolation, sequencing and expression of a genomic DNA clone of the human GDNF gene. WO93/06116 also describes vectors, host

cells, and culture growth conditions for the expression of GDNF protein product. Additional vectors suitable for the expression of GDNF protein product in *E. coli* are disclosed in published European Patent Application No. EP 0 423 980 ("Stem Cell Factor") published April 24, 1991, the disclosure of which is hereby incorporated by reference. The DNA sequence of the gene coding for mature human GDNF and the amino acid sequence of the GDNF is shown in Figure 19 (SEQ ID NO:5) of WO93/06116. Figure 19 does not show the entire coding sequence for the pre-pro portion of GDNF, but the first 50 amino acids of human pre-pro GDNF are shown in Figure 22 (SEQ ID NO:8) of WO93/06116.

Naturally-occurring GDNF is a disulfide-bonded dimer in its biologically active form. The material isolated after expression in a bacterial system is essentially biologically inactive, and exists as a monomer. Refolding is necessary to produce the biologically active disulfide-bonded dimer. Processes for the refolding and naturation of the GDNF expressed in bacterial systems are described in WO93/06116. Standard *in vitro* assays for the determination of GDNF activity are described in WO93/06116 and in co-owned, co-pending U.S. Patent Application Serial No. 08/535,681 filed September 28, 1995, and are hereby incorporated by reference.

A. GDNF variants

The term "GDNF variants" as used herein includes polypeptides in which amino acids have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants"), residues within the amino acid sequence of naturally-occurring GDNF. Such variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the polypeptide or by *in vitro* chemical synthesis of the desired polypeptide. It will be appreciated by those skilled in the art that many combinations of deletions, insertions, and substitutions can be made provided that the final molecule possesses GDNF biological activity.

Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues are well known to one skilled in the art (e.g., U.S. Patent Number 4,518,584, the disclosure of which is hereby incorporated by reference.) There are two principal variables in the construction of variants: the location of the mutation site and the nature of the mutation. In designing GDNF variants, the selection of the mutation site and nature of the mutation will depend on the GDNF characteristic(s) to be modified. The sites

for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target amino acid residue, or (3) inserting amino acid residues adjacent to the located site. Conservative changes in from 1 to 20 amino acids are preferred. Once the amino acid sequence of the desired GDNF protein product is determined, the nucleic acid sequence to be used in the expression of the protein is readily determined. N-terminal and C-terminal deletion variants may also be generated by proteolytic enzymes.

For GDNF deletion variants, deletions generally range from about 1 to 30 residues, more usually from about 1 to 10 residues, and typically from about 1 to 5 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions may be introduced into regions of low homology with other TGF-ß superfamily members to modify the activity of GDNF. Deletions in areas of substantial homology with other TGF-ß superfamily sequences will be more likely to modify the GDNF biological activity more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of the GDNF protein product in the affected domain, e.g., cysteine crosslinking. Non-limiting examples of deletion variants include truncated GDNF protein products lacking from one to forty N-terminal amino acids of GDNF, or variants lacking the C-terminal residue of GDNF, or combinations thereof, as described in co-owned, co-pending U.S. Patent Application Serial No. 08/535,681 filed September 28, 1995, which is hereby incorporated by reference.

For GDNF addition variants, amino acid sequence additions typically include N-and/or C-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as internal intrasequence additions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 10 residues, more typically from about 1 to 5 residues, and usually from about 1 to 3 amino acid residues. Examples of N-terminal addition variants include GDNF with an N-terminal methionyl residue (an artifact of the direct expression of GDNF in bacterial recombinant cell culture), which is designated [Met^{-1}]GDNF, and fusion of a heterologous N-terminal signal sequence to the N-terminus of GDNF to facilitate the secretion of mature GDNF from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Additions may also include amino acid sequences

derived from the sequence of other neurotrophic factors. A preferred GDNF protein product for use according to the present invention is the recombinant human [Met^{-1}]GDNF.

GDNF substitution variants have at least one amino acid residue of the GDNF amino acid sequence removed and a different residue inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. Examples of substitution variants (see, e.g., SEQ ID NO: 50) are disclosed in co-owned, co-pending U.S. Patent Application Serial No. 08/535,681 filed September 28, 1995, and are hereby incorporated by reference.

Specific mutations of the GDNF amino acid sequence may involve modifications to a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of an O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) result in non-glycosylation at the modified tripeptide sequence. Thus, the expression of appropriate altered nucleotide sequences produces variants which are not glycosylated at that site. Alternatively, the GDNF amino acid sequence may be modified to add glycosylation sites.

One method for identifying GDNF amino acid residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science*, 244:1081-1085, 1989). In this method, an amino acid residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing additional or alternate residues at the sites of substitution. Thus, the target site for introducing an amino acid sequence variation is determined, alanine scanning or random mutagenesis is

conducted on the corresponding target codon or region of the DNA sequence, and the expressed GDNF variants are screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in GDNF proteins from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites of interest are those in which particular residues of GDNF-like proteins, obtained from various species, are identical. Such positions are generally important for the biological activity of a protein. Initially, these sites are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes (exemplary substitutions) are introduced, and/or other additions or deletions may be made, and the resulting products screened for activity.

TABLE 1
Amino Acid Substitutions

Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu: Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu: Met; Phe; Ala; norleucine

Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleic acid sequences) are expected to produce GDNF protein products having functional and chemical characteristics similar to those of natural GDNF. In contrast, substantial modifications in the functional and/or chemical characteristics of GDNF protein products may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr;
3) acidic: Asp, Glu;
4) basic: Asn, Gln, His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for another. Such substituted residues may be introduced into regions of the GDNF protein that are homologous with other TGF-ß superfamily proteins, or into the non-homologous regions of the molecule.

B. <u>GDNF Derivatives</u>

Chemically modified derivatives of GDNF or GDNF variants may be prepared by one of skill in the art given the disclosures herein. The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or,

more preferably, by injection or infusion, or, further formulated for oral, pulmonary or other delivery routes), and determining its effectiveness.

Suitable water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight ranges from about 2 kDa to about 100 kDa for ease in handling and manufacturing (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of polyethylene glycol on a therapeutic protein or variant).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono-, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. See for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et

al., *Exp. Hematol.*, 20:1028-1035, 1992 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group is preferred. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire an N-terminal chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the e-amino group of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single

reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention contemplates the use of derivatives which are prokaryote-expressed GDNF, or variants thereof, linked to at least one polyethylene glycol molecule, as well as use of GDNF, or variants thereof, attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage.

Pegylation may be carried out by any of the pegylation reactions known in the art. See, for example: *Focus on Growth Factors*, 3 (2):4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to pegylation. The pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer).

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol with the GDNF protein or variant. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of GDNF protein or variant. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide. As used herein, "acylation" is contemplated to include without limitation the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See *Bioconjugate Chem.*, 5:133-140, 1994. Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions of temperature, solvent, and pH that would inactivate the GDNF or variant to be modified.

Pegylation by acylation will generally result in a poly-pegylated GDNF protein or variant. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., > 95%) mono-, di- or tri-pegylated. Some species with higher degrees of pegylation, however, may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the GDNF protein or variant in the presence of a reducing agent. Pegylation by alkylation can also result in poly-pegylated GDNF protein or variant. In addition, one can manipulate the reaction conditions to favor pegylation substantially only at the a-amino group of the N-terminus of the GDNF protein or variant (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a -CH2-NH- group. With particular reference to the -CH2- group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. The reaction is performed at a pH which allows one to take advantage of the pKa differences between the e-amino groups of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention contemplates use of a substantially homogeneous preparation of monopolymer/GDNF protein (or variant) conjugate molecules (meaning GDNF protein or variant to which a polymer molecule has been attached substantially only (i.e., > 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also encompasses use of pegylated GDNF protein or variant lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the GDNF protein or variant.

Thus, it is contemplated that GDNF protein products to be used in accordance with the present invention may include pegylated GDNF protein or variants, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, and preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the a- or e-amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.